aliphatic and aromatic residues in the binding site of RgSBP and that these residues are involved in the binding of 2,7-anhydro-Neu5Ac.

R. gnavus Sialic Acid Aldolase is Specific for Neu5Ac

[0103] The first step of sialic acid metabolism is the conversion of sialic acid to ManNAc and pyruvate catalysed by a sialic acid aldolase (NanA). To determine the substrate specificity of RUMGNA_02692 (RgNanA), the corresponding gene was amplified by PCR from the *R. gnavus* ATCC 29149 genome, cloned into the pHISTEV expression vector, heterologously expressed in *E. coli* and the His_6-tag recombinant protein purified by immobilised metal ion affinity chromatography (IMAC). The substrate specificity of RgNanA was determined using a coupled activity assay where pyruvate released during the conversion of sialic acid to ManNAc is converted to lactate by a lactate dehydrogenase and the subsequent loss of absorbance at 340 nm measured as NADH is converted to NAD^+. A commercially available *E. coli* sialic acid aldolase (EcNanA) was included as a control and enzymes were tested for activity against 2,7-anhydro-Neu5Ac and Neu5Ac. Both enzymes showed activity against Neu5Ac whilst neither enzyme showed activity against 2,7-anhydro-Neu5Ac (FIG. **7***a*). The products of these reactions were analysed by HPLC and confirmed that reactions of both enzymes with Neu5Ac produced ManNAc, whereas no reaction product was detected when 2,7-anhydro-Neu5Ac was used as a substrate. The kinetic parameters of RgNanA were determined by calculating the initial rate of reaction with increasing Neu5Ac concentrations. A Michaelis-Menten curve was fitted to the data and kinetic parameters determined (FIG. **7***b*). The k_{cat} was calculated at 2.757±0.033 s^{-1} and the K_M 1.473±0.098 mM. These values are consistent with other reported data of sialic acid aldolases in bacteria.

[0104] The crystal structure of RgNanA wt presents as a (β/α8) TIM barrel with an adjacent three-helix bundle, a fold shared with other bacterial Neu5Ac lyases (Barbosa et al., 2000; Huynh et al., 2013; Timms et al., 2013; North et al., 2016; Campeotto et al., 2018; Kumar et al., 2018). Structural inspection of the RgNanA active site indicates a high degree of similarity with previously characterised sialic acid aldolases (FIG. **7***c*), supporting RgNanA substrate specificity for Neu5Ac. The RgNanA wt crystals dissolved in Neu5Ac soaking experiments, as also observed previously with *Pasteurella multocida* Neu5Ac aldolase (Huynh et al., 2013) (PcNanA), which may be due to subtle conformational changes during substrate binding or catalysis. However, following soaking of RgNanA K167A crystals with Neu5Ac, clear electron density for Neu5Ac in the open-chain ketone form was present. Neu5Ac was shown to form extensive interactions with the enzyme active site, with hydrogen bonds to the side chains of Ser49, Ser50, Ser169, Asp194, Glu195, and Tyr257, and main chain atoms of Ser50, Gly192, Asp194, Gly211. The N-acetyl group is oriented out of RgNanA active site. In the active site of the *E. coli* Neu5Ac lyase/aldolase (EcNanA), Ser47, Tyr110, Tyr137, and Thr167 were identified to be important for catalytic activity (Daniels et al., 2014). These residues are conserved in RgNanA with the exception of *E. coli* Thr167, which is Ser169 in RgNanA. The EcNanA Thr167 and RgNanA Ser169 hydroxyls superimpose. Notably, the EcNanA T167S mutation did not affect the enzyme kinetic parameters (ref). Comparing the active sites of the wild type and mutant RgNanA protein highlights a 1.8 Å shift by the Tyr139 α-carbon. This movement is also present in the apo crystal structure, therefore presumably due to the absence of Lys167 rather than the presence of Neu5Ac.

RUMGNA_02695 Catalyses the Conversion of 2,7-anhydro-Neu5Ac to Neu5Ac

[0105] To identify the substrate of RUMGNA_02695, the corresponding gene was amplified by PCR from the *R. gnavus* ATCC 29149 genome, cloned into the pHISTEV expression vector, heterologously expressed in *E. coli* and the His_6-tag recombinant protein purified by immobilised metal ion affinity chromatography (IMAC). The protein is predicted to include a Rossman fold, so the recombinant protein was incubated with 2,7-anhydro-Neu5Ac in the presence and absence of NAD^+/NADH/FAD as potential cofactors. The products of each reaction were analysed by HPLC following DMB labelling of the sialic acid as reported previously (Monestier et al., 2017). Neu5Ac was observed as a reaction product when the enzyme was incubated with 2,7-anhydro-Neu5Ac in the presence of NAD^+ or NADH, but not in the presence of FAD or in the absence of a cofactor (FIG. **8***a*).

[0106] Mass spectrometry (MS) was further used to monitor the enzymatic reaction. These analyses showed a ratio of 1:2 for 2,7-anhydro-Neu5Ac:Neu5Ac, suggesting that the reaction may be reversible. To test this further, the recombinant enzyme was incubated with Neu5Ac in the presence of NAD^+/NADH, and the reaction products analysed by MS. The 2,7-anhydro-Neu5Ac to Neu5Ac ratio was approximately 1:2, confirming that the reaction is reversible, with Neu5Ac as the favourable product. To investigate the role of the cofactors (NAD^+ or NADH) in the enzymatic reaction, the concentration of NADH was determined by monitoring the absorbance at 340 nm for reactions using 2,7-anhydro-Neu5Ac or Neu5Ac as substrate. No change in absorbance was detected, suggesting that the enzyme mechanism may involve oxidation and reduction of NADH cofactor. Since no net change in NADH concentration was observed during the conversion of 2,7-anhydro-Neu5Ac to Neu5Ac by RUMGNA_02695, the kinetic parameters of the enzymatic reaction were determined using the coupled reaction described above. Here, the reaction catalysed by RUMGNA 02695 was carried out in the presence of an excess of aldolase and increasing concentrations of 2,7-anhydro-Neu5Ac substrate (FIG. **8***b*). Using these conditions, the k_{cat} was calculated to be 0.0824±0.0043 s^{-1} and the K_M 0.074±0.014 mM.

[0107] Taken together these data indicate that RUMGNA_02695 is a novel oxidoreductase required for the conversion of 2,7-anydro-Neu5Ac into Neu5Ac, which then becomes a substrate for

[0108] RgNanA. We will refer to RUMGNA_02695 as RgNanOx in the rest of the study.

The Nan Cluster is Essential for *R. gnavus* to Utilise Sialoconjugates or 2,7-anhydro-Neu5Ac In Vitro

[0109] The ClosTron transformation method (Heap et al., 2010) was successfully applied to *R. gnavus* ATCC 29149 for the first time, enabling the generation of nan deletion mutants with an erythromycin resistance gene present in